SUPPORTING INFORMATION

Intraduplex DNA-mediated electrochemistry of covalently tethered redox-active reporters

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Materials

Chemicals and reagents used in the preparation of the activated redox active reporters were purchased from Sigma and used without further purification. MB′ was prepared based on previously established protocols (1). All phosphoramidites and DNA synthesis reagents were purchased from Glen Research. Silicon wafers used for the fabrication of multiplexed chips were purchased from SiliconQuest.

Synthesis of proprionic acid modified Nile Blue (NB′).

See Supporting Scheme 1 for the synthetic strategy for the preparation of **NB′**.

*5-(Dimethylamino)-2-nitrosophenol (**1**) preparation*. **1** was prepared according to the procedure described by Moura (2) by dissolving 3-(dimethylamino)phenol (8.26 g, 50 mmol) in concentrated hydrochloric acid (25 mL) chilled to 0 ºC followed by the slow addition of sodium nitrite (4.0g, 58 mmol). The reaction mixture was maintained at 0 ºC and the addition of sodium nitrite was slowed if orange fumes were observed. The reaction was left to proceed for 30 min – 1 hr, until the contents solidified. The precipitate was isolated by vacuum filtration and washed with chilled diluted hydrochloric acid (1 N). The yellow-brown solid (5.6 g, 29 mmol, 57% yield) was confirmed as the desired product by ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.66-7.24 (m, 1H), 7.24-6.78 (m, 2H), 3.36 (s, 6H) and ESI-MS in 7:3 $CHCl_3$:MeOH by the observed $[M+H]^+$ peak at 167.0 g/mol. **1** was dried and used in following reactions without further purification.

*3-(naphthalen-1-ylamino)propanoic acid (**2**) preparation.* **2** was prepared by dissolving naphthylamine (2.0 g, 14 mmol) in water (15 mL) followed by the addition of sodium hydroxide (6 M, 5.0 mL) and equimolar amounts of dissolved chloroproprionic acid (1.50 g, 15 mmol). The reaction was brought to a reflux and allowed to proceed for 12-24 hours. The excess solvent was removed under reduced pressure and the desired product was purified by silica chromatography ($CHCl_3$:MeOH 20:1) with an R_f of 0.5. The clear fraction was dried to yield a white solid product that was confirmed as the desired product (**2**) by ^{1}H NMR (300 MHz, CD_3OD): δ 80.5-7.87 (m, 1H), 7.85-7.62 (m, 1H), 7.53-7.23 (m, 3H), 7.18 (t, *J* = 8.4 Hz, 1H), 6.65 (t, *J* = 8.0 Hz, 1H), 3.59 (dt, *J* = 9.4, 7.0 Hz, 2H), 2.75 (t, 2H) and ESI-MS in 7:3 $CHCl_3$:MeOH by the observed $[M+H]^+$ peak at 216.1 g/mol.

*Proprionic acid modified Nile Blue (**NB′**) preparation*. **NB′** was prepared based on previously established procedures by Moura (3). Equimolar portions of **1** (0.17 g, 1.0 mmol) and **2** (0.22 g, 1.0 mmol) were combined and dissolved in dimethylformamide (DMF) (6 mL). The reaction mixture was heated to 70 ºC for 24 hours. The crude reaction mixture was dried under reduced pressure and purified by dry silica chromatography (4). Pure **NB′** was eluted with 6:1 cholorform: methanol as a dark blue compound. The identity was verified by ESI-MS in MeOH:H_2O 5:1 based on the observed $[M]^+$ peak at 362.3 g/mol.

NHS-ester activation of carboxylic acid modified reporters

MB′, NB′, and anthraquinone-2-carboxylic acid (AQ′) were all activated to the NHS-esters immediately prior to coupling to amino-modified DNA to aid the yield of amide bond formation. The same procedure as previously used for the activation of MB′ was applied to the activation of all three of these redox active reporters (1). Briefly, the carboxylic acid modified reporter (0.022 mol) was stirred at room temperature in DMF with excess N,N′-dicyclohexylcarbodiimide (9.3 mg, 0.045 mmol) and N-hydroxysuccinimide (5.2 mg, 0.045 mmol) for 12-24 hours. The solvent was subsequently removed under reduced pressure and the reaction mixture was re-suspended in DMSO (20 µL per 1 mg activated).

Preparation of probe and thiol modified oligonucleotides

Thiol-modified and amino-modified DNA were synthesized and purified based on previously established protocols (1, 5). The procedure for DNA purification was unaltered across all three different amino modifiers utilized: dT-C6, dT-C4, and 5′-C6. The purified amino-modified DNA was covalently modified with NHS-ester activated MB′, NB′, and AQ′ using roughly 10-fold excess reporter in an aqueous basic solution (0.1 M $NaHCO_3$). The same thiol-modified complement stand was used for all duplex DNA, to negate variations caused by the thiol driven self-assembly of the DNA. Both well-matched and AC mismatched sequences were prepared of a CG-rich 17-mer duplex to investigate the effect due to π-stack perturbations (Supporting Table 1).

Supporting Table 1. DNA sequences used for electrochemical measurements

Name	Sequence
Thiol-ssDNA	5'- HS - C_6 – GAC TGA CCT CGG ACG CA -3'
Well-matched ssDNA (WM) [a, b]	5'-**<u>T</u>**G CGT CCG AGG TCA GTC -3'
AC-mismatched ssDNA (MM) [a, b]	5'-**<u>T</u>**G CGT CC*A* AGG TCA GTC -3'

[a] **<u>T</u>** is the site of the amino-modified nucleotide used to produce the dT-C6 and dT-C4 linkages.
[b] 5′-C6 linkage is produced by an additional 5′ phosphoramidite.

Electrochemistry of DNA-modified electrodes

Multiplexed chips, with 16 individually addressable electrodes, were prepared and used based on previously established protocols (6, 10). These 16-electrodes were divided into 4 isolated quadrants, each with 4 electrodes, allowing for 4 different typical of DNA or morphologies to be simultaneous compared without any variability introduced due to changing the underlying gold surface. Electrodes were exposed to duplex thiol-modified DNA (20 μL of 25 μM) in phosphate buffer (5.0 mM phosphate, 50 mM NaCl, pH 7) and allowed to self-assemble for 12-16 hours in a humid environment. $MgCl_2$ was added to solutions during DNA self-assembly as indicated in the text to alter the immobilization of the DNA. After DNA self-assembly, electrodes were washed with excess phosphate buffer and incubated with 6-mercaptohexanol (1 mM for 45 min) in phosphate buffer with 5% glycerol. Electrodes were washed again with excess phosphate buffer.

Electrochemical measurements were performed with a CHI620D Electrochemical Analyzer and a 16-channel multiplexer from CH Instruments. A three-electrode setup was used with a common Pt auxiliary and a quasi Ag/AgCl reference electrode (Cypress Systems) placed in the central well of the clamp. Cyclic voltammetry data were collected at 100 mV/s over a window of 0 mV to -0.6 mV versus Ag/AgCl unless otherwise indicated. Every scan consisted of 8 sweeps of the potential window (4 reductive and 4 oxidative) and the last two sweeps are used for quantification and to present all data. Only signals that were stable across repetitive scans and were distinct to the given reporter were used for this investigation. The electrodes were exposed to the various running conditions in the following sequence: an initial background scan acquired in phosphate buffer (5.0 mM phosphate, 50 mM NaCl, pH 7), DNA quantification by $Ru(NH_3)_6^{3+}$ (1 μM in phosphate buffer), excess washing with phosphate buffer until $Ru(NH_3)_6^{3+}$ signal is no longer present, final scan in spermidine buffer (5.0 mM phosphate, 50 mM NaCl, 4 mM $MgCl_2$, 4 mM spermidine, 50 µM EDTA, 10% glycerol, and pH 7.0). Unless otherwise indicated presented and quantified data for reporter signals is determined from CV in spermidine buffer. The signal averages and associated error are for a given data set (comparison of reporters vs. comparison of linkages) has many other factors that can alter the signal size including surface and self-assembly quality. To control for this, the data was acquired in distinct data sets such that results being directly compared are always from multiplexed chips running in close succession. Therefore, the data presented is either quadrant averages (4 electrodes) or an average of *n* quadrants (*n* x 4 electrodes) acquired within no more than a few days of each other and is indicated where applicable.

The Laviron analysis was used to determine the electron-transfer rate constant (*k*) for MB-modified DNA (7, 8). A plot of the peak shift ($E_{pc} - E^{o'}$, mV), where E_{pc} is the peak potential at a given scan rate and $E^{o'}$ is the midpoint potential determined at 50 mV, versus the ln(scan rate), where the scan rate (*v*, mV/s) is varied from 50 mV/s to 13 V/s was constructed. The electron transfer rate can then be derived from the linear portion of this plot (ΔE vs ln(*v*), see Figure S5) based on Equation 1. However, the rates determined using this analysis are useful for comparisons and can only be assessed with confidence as order of magnitude estimates.

Equation 1. Formula used to determine the rate of reduction (k, s^{-1}) based on the Laviron anaylsis (7, 8).

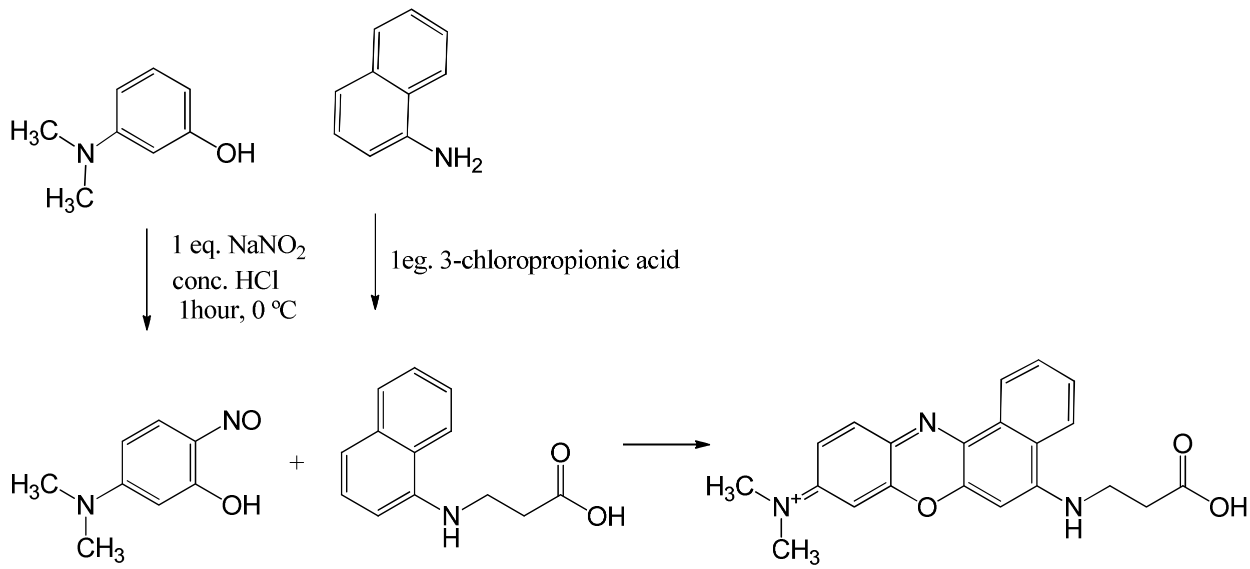
$$E_{pc} = E^{o'} - \frac{RT}{\alpha nF} \ln\left(\frac{\alpha nFv}{RTk}\right)$$

Quantification of immobilized DNA

The quantity of immobilized DNA within a given DNA monolayer was quantified based off the area of reductive signal generated from the electrostatic binding of $Ru(NH_3)_6^{3+}$. A concentration of 1 mM $Ru(NH_3)_6^{3+}$ was chosen based on a titration of both DNA surface coverages and $Ru(NH_3)_6^{3+}$ concentrations. The surface coverage of DNA (Γ) was calculated using equation 1 where *n* is the number of electrons per reduction event, *F* is the Faraday constant, *A* is the electrode area in cm^2 (2 cm^2), *z* is the charge on the $Ru(NH_3)_6^{3+}$, *m* is the number of base in the duplex DNA (17 mer), and finally *Q* is the reductive signal from $Ru(NH_3)_6^{3+}$ in phosphate buffer minus the redox reporter signal in spermidine buffer. As the contribution of reporter reduction could not be deconvoluted from the $Ru(NH_3)_6^{3+}$ signal it was subtracted even though it was consistently negligible and less than 10% of the total $Ru(NH_3)_6^{3+}$ signal. Therefore the Q_{Ru} used for determining the surface coverage is obtained by taking the area of the $Q_{Ru + MB}$ and subtracting out the Q_{MB} (obtained after washing off the $Ru(NH_3)_6^{3+}$).

Equation 2. Formula used to determine the surface coverage (Γ, pmol/cm^2) of immobilized DNA based on $Ru(NH_3)_6^{3+}$ binding.

$$\Gamma_{DNA} = \frac{Q_{Ru}}{n\,F\,A}\,\frac{z}{m}\;N_A$$



1 **2** **NB'**

Supporting Scheme 1. Synthetic strategy for the preparation of proprionic acid modified Nile Blue (NB′).

Supporting Table 2. Thermal Stability of Duplex DNA [a]

Redox Active Species	T_M (°C)	Stabilization
Methylene Blue	63.7 °C	+ 1.6 °C
Nile Blue	63.1 °C	+ 1 °C
Anthraquinone	62.3 °C	+ 0.2 °C
None	62.1 °C	-

[a] Duplex DNA (1 µM) was incubated with the redox active species (5 µM) in phosphate buffer (5.0 mM phosphate, 50 mM NaCl, pH 7.0) and the absorbance at 260 nm was monitored every 0.5 °C from 25 - 90 °C. The maximum of the derivative of the absorbance temperature trace was determined to extract the melting temperature under each condition.

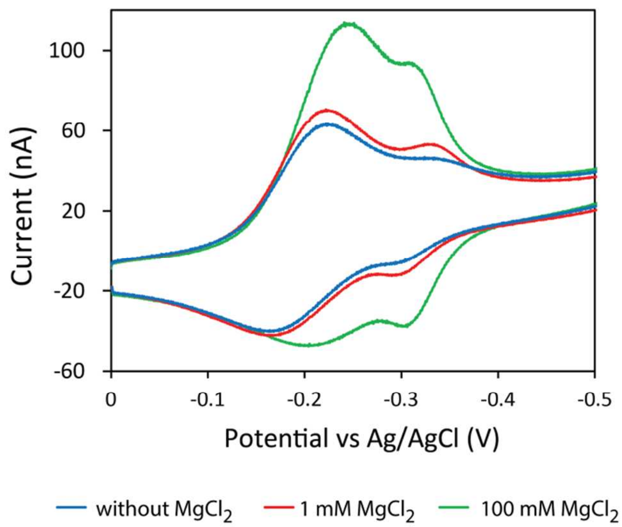


Figure S1. Electrostatic binding of $Ru(NH_3)_6^{3+}$ to immobilized DNA. Representative cyclic voltammograms (scan rate = 100 mV/s) of MB-dT-C12-DNA-modified electrodes acquired after the addition of $Ru(NH_3)_6^{3+}$ (1 μM) in phosphate buffer (5.0 mM sodium phosphate, 50 mM NaCl, pH 7.0) are presented. The concentration of $MgCl_2$ present during DNA self-assembly was varied to alter the overall DNA morphology: without $MgCl_2$ (blue), with 1 mM $MgCl_2$ (red), and with 100 mM $MgCl_2$ (green). The representative traces for the three different DNA morphologies were obtained on a single multiplexed chip with a common $Ru(NH_3)_6^{3+}$ solution. The area of the total reductive signal minus the area of the reporter reductive signal, acquired after washing off the $Ru(NH_3)_6^{3+}$, was used to determine the surface coverage of DNA on the electrode as outlined above.

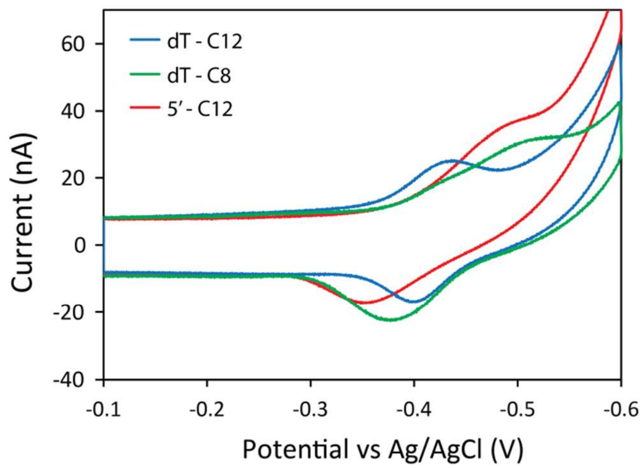


Figure S2. Variation of covalent linkage for NB-DNA. Cyclic voltammetry (scan rate = 100 mV/s), in spermidine buffer (5.0 mM phosphate, 50 mM NaCl, 4 mM $MgCl_2$, 4 mM spermidine, 50 μM EDTA, 10% glycerol, and pH 7.0), of NB covalently tethered to duplex DNA via three different linkages: 5′-C12 (red), dT-C12 (blue), and dT-C8 (green). The monolayers of 17-mer well-matched DNA were assembled in the presence of 100 mM $MgCl_2$ to yield high density DNA films. See Supporting Table 1 for sequences and Figure 2 for structures of linkages.

Supporting Table 3. Electrochemical parameters for MB signals

Linkage	Mid-point Potential (mV)	Peak Splitting (mV)	Signal Size (nC) [a]
dT-C12	-300	35	9.1
dT-C8	-330	124	8.4
5′-C12	-280	26	10.2

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[a] Signal sizes were determined from the average of 4 electrodes within a quadrant on a given multiplexed chip. The data used to compare all three linkages was from the same multiplexed chip and the same thiol strand was used to help negate surface and self-assembly affects.

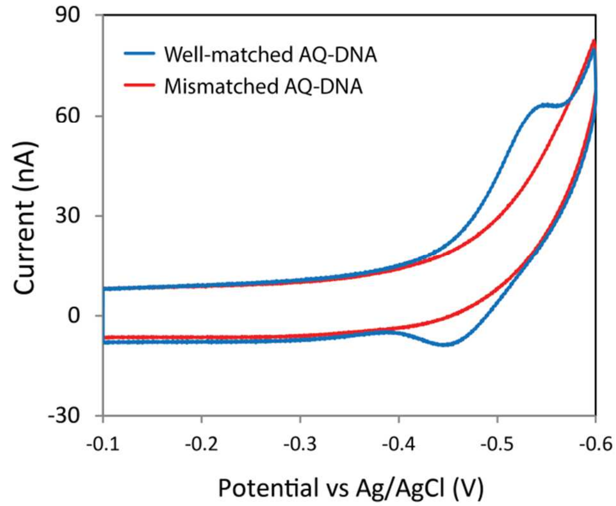

Figure S3. AQ-dT-C12-DNA response to π-stack perturbations. Cyclic voltammograms (scan rate = 100 mV/s) of AQ-dT-C12-DNA modified electrodes self-assembled in the presence of 100 mM $MgCl_2$ and acquired in de-oxygenated spermidine buffer (5.0 mM phosphate, 50 mM NaCl, 4 mM $MgCl_2$, 4 mM spermidine, 50 μM EDTA, 10% glycerol, and pH 7.0). The sensitivity to the introduction of a single perturbation to the π-stack is demonstrated by the direct comparison of 17-mer well-matched (blue) and AC mismatched (red) DNA (see Supporting Table 1 for sequences). Despite de-oxygenation of the multiplexed chips, the extent of the background signals at the negative potentials of AQ allowed for only qualitative comparisons to be performed.

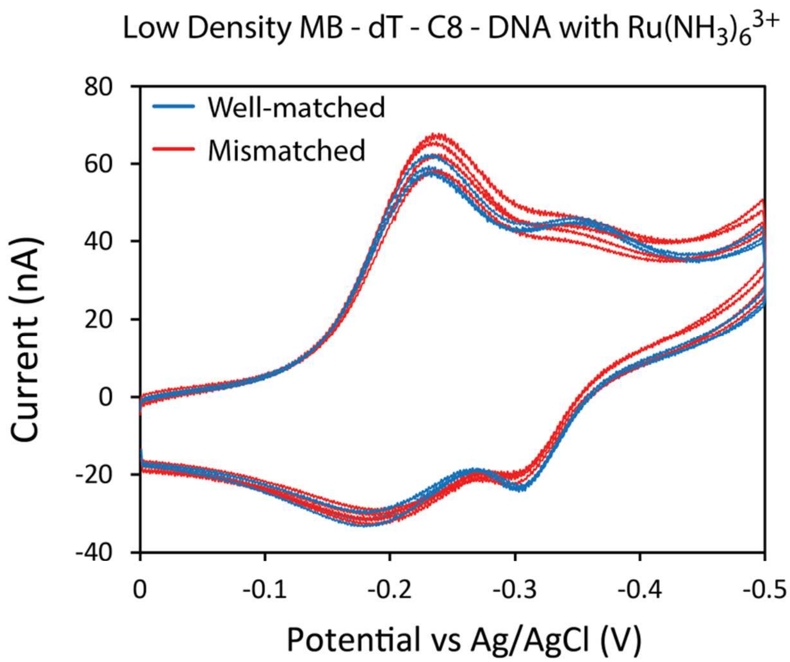

Figure S4. Consistency of $Ru(NH_3)_6^{3+}$ quantification across DNA sequences. Cyclic voltammograms (CVs) of the quantification of immobilized DNA by the electrostatic binding of $Ru(NH_3)_6^{3+}$ (1 μM) in phosphate buffer are presented for MB-dT-C8-DNA. CVs for low density DNA monolayers and both well-matched (blue) and AC mismatched (red) 17-mer DNA are presented. For each given condition 4 individual electrodes are presented to demonstrate the variability observed. Overall, the DNA sequence is shown to have no significant impact on the signal from $Ru(NH_3)_6^{3+}$ binding indicating that there is not significant de-hybridization of the mismatched duplex at the surface of the electrode.

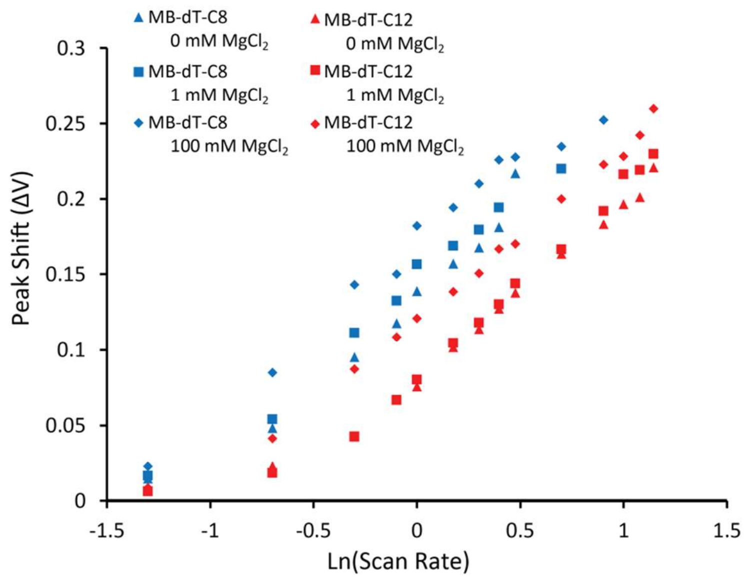

Figure S5. Scan rate dependence. The scan rate was varied from 50 mV to 13 V and the shift in the reduction potential was determined relative to midpoint potential quantified at 50 mV for MB covalently tether to DNA by both dT-C8 (blue) and dT-C12 linkages (red). Three different assembly conditions were also examined for each linkage: without $MgCl_2$ (triangle), with 1 mM $MgCl_2$ (square), and with 100 mM $MgCl_2$ (diamond). Cyclic voltammetry was acquired in spermidine buffer (5.0 mM phosphate, 50 mM NaCl, 4 mM $MgCl_2$, 4 mM spermidine, 50 μM EDTA, 10% glycerol, and pH 7.0) with well-matched DNA. The rate of electron transfer was determined based on the Laviron analysis (7, 8) and was found to be 2 - 4 s^{-1} for all conditions.

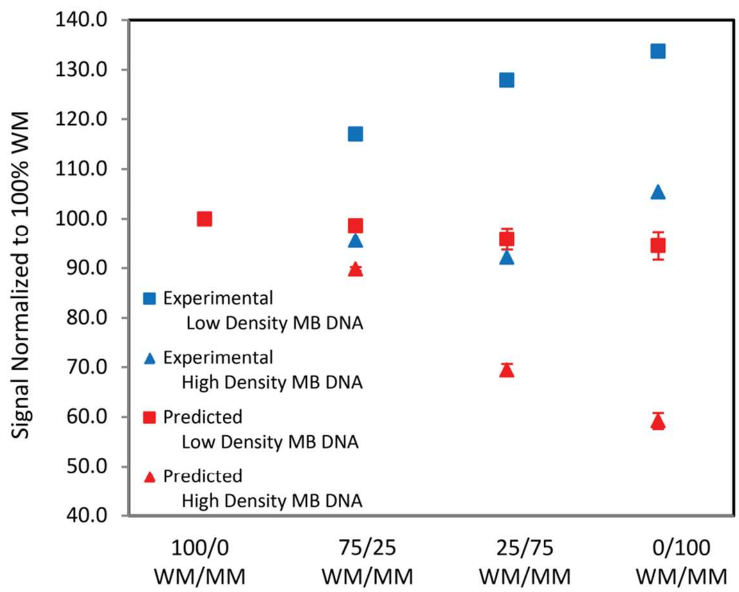

Figure S6. Effect of neighboring duplex integrity for MB-dT-C12-DNA. Electrodes assembled with MB-modified well-matched DNA and varied fractions of unlabeled well-matched and AC mismatched DNA. The predicted (red) and experimental (blue) reductive signal areas were determined at each fraction of unlabeled mismatched DNA and normalized to the signal at 100% well-matched DNA. Electrodes assembled in the presence (triangle) and absence (square) of 100 mM $MgCl_2$ are presented.

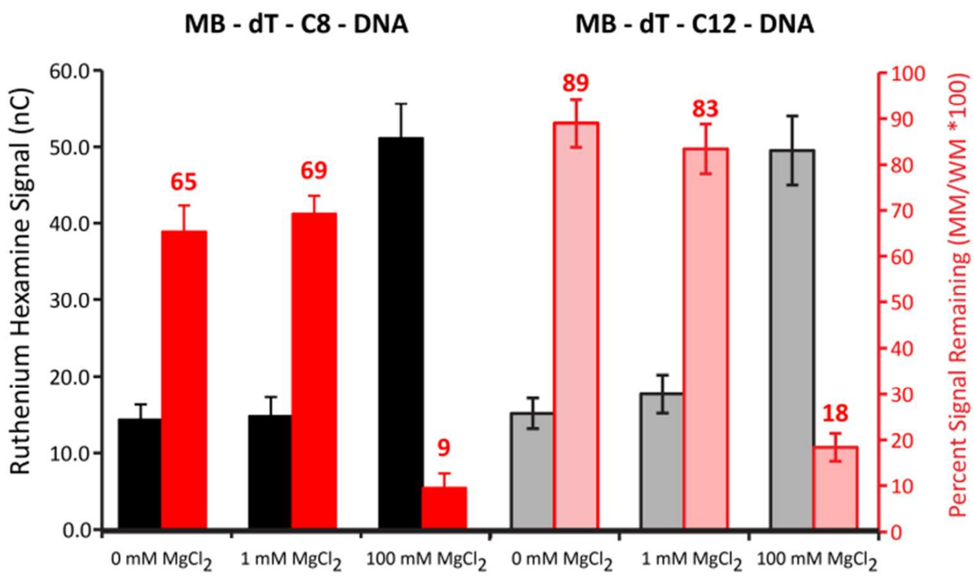

Figure S7. Electrochemistry as a function of assembly conditions. The percent signal remaining (MM/WM*100) (red) and Ru(NH3)63+ signals (black) for MB modified DNA with both dT-C8 (dark) and dT-C12 (light) linkages were examined. DNA-modified electrodes were assembled without MgCl2, with 1 mM MgCl2, and with 100 mM MgCl2. The error was determined from across 3 sets each containing 4 electrode replicates.

References

1. Pheeney, C.G.; Barton, J. K. *Langmuir* **2012**, *28*, 7063.
2. Frade, V.H.J.; Gonçalves, M.S.T.; Moura, J.C.V.P. *Tetrahedron Letters* **2006**, *47*, 8567.
3. Frade, V.H.J.; Gonçalves, M.S.T.; Moura, J.C.V.P. *Tetrahedron Letters* **2005**, 46, 4949.
4. Pedersen, D.S.; Rosenbohm, C. *Synthesis* **2001**, *16*, 2431.
5. Slinker, J.D; Muren, N.B.; Gorodetsky, A.A.; Barton, J.K. *J. Am. Chem. Soc.* **2010**, *132*, 2769.
6. Pheeney, C.G.; Arnold, A.R.; Grodick, M.A.; Barton, J.K. *J. Am. Chem. Soc.* **2013,** *135*, 11869.
7. Laviron, E. J. *J. Electroanal. Chem.* **1979**, *101*, 19.
8. Yu, H-Z.; Luo, C-Y.; Sankar, C.G.; Sen, D. *Anal. Chem.* **2003**, *75*, 3902.